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Keratin 19 protein expression is an independent predictor of survival in human hepatocellular carcinoma

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Disclosures/Conflict of Interest

None declared

Abstract

Aims: We aimed to assess the clinico-pathological relevance and prognostic significance of hepatic progenitor cell markers keratin 19 (K19), Epithelial Cell Adhesion Molecule (EpCAM) and CD117 (c-KIT) expression in a Caucasian series of hepatocellular carcinoma (HCC).

Methods: We evaluated the immunohistochemical expression of K19, EpCAM and CD117 in 89 surgical specimens of HCC from Greek patients (mean age 66.7 ± 11.3 years, male 75.2%) followed-up for 39.6 ± 25.3 months.

Results: K19-, EpCAM- and CD117-specific expression was detected in tumour cells of 10.11%, 15.38% and 3.7% HCCs, respectively. Female gender was correlated with EpCAM immunohistochemical expression ($p=0.035$), while no other significant relationship with clinico-pathological parameters was observed. K19-positivity tended to correlate with microvascular invasion ($p=0.054$). In univariate analysis, K19-positivity and microvascular invasion were associated with decreased recurrence-free (RFS) ($p<0.001$ and $p=0.004$, respectively) and overall survival (OS) ($p=0.002$ and $p=0.029$, respectively). EpCAM- and CD117-positivity did not correlate with patient survival. In multivariate analysis, K19-positivity emerged as an independent predictor of RFS (OR=7.84, 95%CI=2.658-22.912; $p<0.001$) and OS (OR=3.845, 95%CI=1.401-10.549; $p=0.009$).

Conclusions: Our study confirms the prognostic significance of K19 expression in Caucasian HCC providing further evidence that it may be used to stratify HCC according to tumour aggressiveness.

Keywords

Hepatocellular carcinoma, hepatic progenitor cells, keratin 19, Epithelial Cell Adhesion Molecule, CD117, c-KIT, recurrence-free survival, overall survival

Introduction

Hepatocellular carcinoma (HCC) is a neoplasm of increasing incidence and is the third cause of cancer-related death worldwide [1]. HCC is currently considered a heterogeneous entity due to substantial differences in molecular profile and biological behaviour. HCC diversity also refers to the cells of origin since mature hepatocytes as well as hepatic progenitor cells (HPC) may be the target population in hepatocarcinogenesis within the context of advanced liver disease and cirrhosis [2]. Direct proof that primary liver carcinomas with mixed features derive from HPC/oval cells originate only from rodent studies [3], while indications from human studies were until recently limited and/or speculative. The strongest evidence to date that liver malignancy in humans may derive from HPC has been the demonstration of HPC/stem cell marker expression in HCC and the associated worse patient prognosis [4, 5].

Carcinomas of HPC origin and their subtypes are grouped together into the category of combined hepatocellular-cholangiocarcinoma according to World Health Organisation (WHO) 2010 classification of liver tumours [6]. Another recently proposed HCC subtype of assumed HPC origin is emerging defined by the presence of a tumour cell fraction expressing HPC markers, but not otherwise recognisable by routine haematoxylin-eosin (H-E) stain [7]. Several markers, including keratin 19 (K19), epithelial cell adhesion molecule (EpCAM), CD133, CD117 (c-KIT) and SALL4 have been suggested as indicators of HPC origin in HCCs [8]. K19, normally found in HPCs and cholangiocytes but not in hepatocytes, has been proven to be the most reliable marker for the characterization of HPCs [9, 10]. Markers of stemness

have been associated with increased telomere length and chromosomal instability [11], microvascular invasion [12], high metastatic potential [13] and poor survival [14-16]. There is also increasing evidence suggesting that K19 immunohistochemical expression may be used as a prognostic marker in human HCC [10, 14, 17-22]. However, the majority of these studies are based on HCC of Asian origin. HCC with a K19-expressing cell population is not as yet an established entity, while controversial data regarding biological behaviour require further investigation at clinico-pathological and molecular level.

Similarly, EpCAM represents a marker for stem cell/progenitor cells of adult human liver and oval cells in rodent liver and can be used to classify HCCs according to tumour aggressiveness. EpCAM expression has been associated with worse outcomes following liver transplantation [23] or resection [9, 24, 25]. Although no association has so far been established with regards to CD117 expression and poor prognosis, CD117 has also been considered as a potential marker of stem/progenitor cells in previous HCC studies [26-30].

The objective of the present study was to examine K19, EpCAM, CD117 expression in a series of human HCCs of Caucasian origin and their potential correlation with clinico-pathological parameters and patient prognosis after curative liver resection or transplantation.

Methods

We retrospectively identified all HCC cases (explant livers and resection specimens) from the archives of the 1st Department of Pathology and the Laboratory of Histology-Embryology, Medical School, National and Kapodistrian University of Athens, between June 1996 and March 2009. We subsequently sought clinical details of these patients through their medical notes including treatment received and overall survival. In total, 113 patients with histologically diagnosed HCC were identified; among these 89 patients had sufficient clinical data and were included in the study. Clinical parameters of the study population were retrospectively collected. Mean patient age was 66.7 ± 11.3 years (range 33-85) and 75.2% were male.

Data on the aetiology of underlying chronic liver disease was available in 72 cases and included hepatitis B virus (HBV) infection ($n=30/72$, 41.7%), hepatitis C virus (HCV) infection ($n=21/72$, 29.2%), alcohol-related ($n=4/72$, 5.6%) and non-alcoholic steatohepatitis ($8/72$, 11.1%). Hepatitis B and C co-infection was present in $5/72$ cases (6.9%). Other aetiologies included haemochromatosis ($n=2$), and primary biliary cirrhosis (PBC) ($n=2$). In 17 cases the underlying aetiology could not be established. Cirrhosis was present in 46 out of the 73 (63%) patients with background non-neoplastic tissue available for analysis. Thirteen patients received loco-regional treatment prior to resection or transplantation (transarterial chemoembolization-TACE $n=12$, radiofrequency ablation-RFA $n=1$).

Pathological variables recorded included tumour size, presence of multiple tumours, tumour grading according to Edmondson-Steiner [31], capsular invasion, microvascular and/or macrovascular invasion, and presence of cirrhosis in the non-neoplastic liver parenchyma. Staging was performed according to TNM classification (7th edition) [32].

The study was performed in accordance with National Bioethical Standards and ethical standards of the revised Helsinki Declaration of World Medical Association (2000). Appropriate consent for use of anonymised archival tissue for research was obtained in each case.

Immunohistochemistry

Formalin-fixed, paraffin-embedded 4µm-thick serial tissue sections of each case were preheated at 56° C, deparaffinised in xylene, rehydrated in graded ethanol, and then washed in phosphate buffered saline (pH=7.6). Heat induced antigen retrieval was performed with 10mM citrate buffer (pH 6.0) for 12 min (for K19 and EpCAM) or with EDTA (pH=9) for 8 min (for CD117) in a microwave oven (600w). Immunohistochemistry was performed using the NovoLink Polymer Detection System (Novocastra Laboratories-Menarini, Athens, Greece) and mouse monoclonal antibodies to K19 (clone A53-B/A.2.26, Thermo Scientific, UK) (1/50 dilution), EpCAM (ESA Ab-7, MOC31, Neomarkers, Freemont, CA, USA) (1/50 dilution), and CD117 (clone 2E4, Zymed, San Francisco, CA, USA) (3/100 dilution) with overnight incubation at 4°C. 3',3'-diaminobenzidine (DAB) was used as chromagen and light haematoxylin as counterstain.

Cholangiocytes in non-neoplastic liver parenchyma present in most sections served as internal positive control for K19 and EpCAM, while mast cells were used as internal positive control for CD117. In negative control sections of each case, the primary antibody was omitted and replaced with antibody diluent (RE7133, Novocastra Laboratories, UK) compatible with the immunohistochemical kit used.

Evaluation of immunohistochemical staining

Immunostained sections were evaluated by two observers (D.T., I.D.), blinded to the clinical and follow-up data, at a double-headed microscope. Positivity for K19, EpCAM or CD117 was defined as specific cytoplasmic and/or membrane immunostaining in $\geq 5\%$ of tumour cells [14, 15, 21, 22, 33]. Immunopositive ductular cells, isolated or in strings, originating from surrounding non-neoplastic liver parenchyma and localized at the tumour periphery (Figure 1) were not counted.

Follow up

Sixty eight out of 89 patients were followed to the date of HCC-related death or to the end of follow-up (31 January 2011). The remaining 21 patients were lost to follow-up. Patients who underwent liver transplantation (n=9) were censored as alive on the day of transplantation. Mean follow-up was 39.6 ± 25.3 months (median 33 months).

Statistical Analysis

Statistical analysis was performed using SPSS software, version 19.0 (SPSS, Chicago, IL) and assessed using the t-test, Pearson's correlation test, or chi-square test, as deemed appropriate. Data were reported as mean \pm standard deviation (SD) if normally distributed and as median if not. Recurrence-free survival (RFS) and overall survival (OS) were calculated using the Kaplan-Meier method, and differences in survival rate were compared using the Log Rank test. Deaths from causes other than HCC were not considered for inclusion in survival analysis. Multivariate analysis for OS and RFS was performed using the Cox-proportional hazards model with backward stepwise selection. Statistical significance was assumed when $p < 0.05$.

Results

Clinical and pathological features of the study population

Data on some clinico-pathological features were not available for all HCC cases (Table 1). Multi-nodular HCC (≥ 3 nodules) was present in 20/75 (26.7%) cases while 37/72 (51.4%) had maximum tumour diameter > 5 cm. Microvascular invasion was observed in 39/70 HCC (55.7%) and 18/47 (38.3%) showed portal vein thrombosis either on imaging or at the time of operation. In 27/54 (50%) and 8/56 (14.3%) HCC, liver capsule invasion or rupture was noted, accordingly. Distant metastasis was recorded in 6/36 (16.7%) patients. Post-surgical staging according to TNM classification was as follows: stage I:24, II:15, III:32 (IIIa:23, IIIb:6, IIIc:3), IV:6 patients. In 12 patients TNM staging could not be assessed due to missing data.

Correlation with clinico-pathological parameters

KERATIN 19

Keratin 19 was positive in 9/89 HCCs (10.11%) (Figures 2A and 2C). K19 immunostaining was noted in the cell membrane and/or cytoplasm of positive tumour cells. The percentage of K19-positive tumour cells ranged from 5% to 90% (mean $24.4 \pm 32.54\%$, median 5%), (Figure 3B) and immunostaining was focal in the majority of cases (7/9). Only 3/9 K19-positive HCC focally showed positive tumour cells with morphological features suggestive of stem/progenitor cells (small, hyperchromatic nuclei and scant cytoplasm). Distinctive histological patterns of HCC (trabecular, solid, pseudoglandular, clear cell etc) did not correlate with K19-positivity. In K19-positive HCC with microvascular invasion, when intravascular tumour cells were included in the immunostained sections these were also K19-positive.

Age, sex, tumour size >5cm, histological grade, TNM staging, presence of cirrhosis, multi-nodular disease, and viral aetiology did not correlate significantly with K19 protein expression ($p > 0.05$). Of all parameters examined, only microvascular invasion tended to correlate with K19 immunohistochemical expression ($p = 0.054$) (Table 1). There were no statistical differences between HCC subgroups with focal or diffuse K19-positivity.

EpCAM

EpCAM expression was assessed in 78 HCC with tissue available for immunostaining (Figures 2B and 2D). EpCAM was positive in 12/78 HCCs (15.38%) (Figure 2B). EpCAM-specific immunostaining was membranous in positive tumour cells. The percentage of EpCAM-positive tumour cells ranged from 26% to 90% (mean 58.17 ± 25.7 , median 58.5%) (Figure 3C) and immunostaining was diffuse in the majority of cases (9/12). Two out of 12 EpCAM-positive HCC focally showed positive tumour cells with morphological features suggestive of stem cells. Distinctive histological patterns of HCC did not correlate with EpCAM-positivity. In EpCAM-positive HCC with microvascular invasion, when intravascular tumour cells were included in the immunostained sections these were also EpCAM-positive.

The correlation between EpCAM expression and clinical or pathological parameters was examined (Table 1). Age, tumour size >5cm, histological grade, presence of cirrhosis, multi-nodular disease, and viral aetiology showed no correlation with EpCAM protein expression ($p > 0.05$). Of all parameters examined, only female gender was significantly correlated with EpCAM immunohistochemical expression ($p = 0.035$). Three cases were positive for both K19 and EpCAM, 9 were K19-negative/EpCAM-positive, 5 were K19-positive/EpCAM-negative, and 61 were negative for both markers. In serial sections of K19-positive/EpCAM-positive HCC, more tumour cells were positive for EpCAM compared to K19 (Figures 2A and 2B). Co-expression of K19 and EpCAM occurred in the minority of tumour cells while the majority expressed only one of the two markers.

CD117

CD117 was positive only in 3/81 (3.7%) cases in the cytoplasm of a small percentage of tumour cells (5%) (Figure 3D). Rare CD117-positive tumour cells showed morphological features of stem cells. CD117 immunopositivity did not show significant correlation with any of the clinico-pathological parameters examined ($p>0.05$) (Table 1). Two grade 3 CD117-positive HCC cases had multifocal disease with vascular invasion (portal vein thrombosis) and none of the cases had distant metastasis. One grade 3 HCC showed co-expression of K19 and CD117 and one grade 2 HCC showed co-expression of EpCAM and CD117.

Survival analysis

During follow-up, 43 out of 68 (63.2%) patients had recurrent disease and 28/68 (41.2%) died of HCC-related causes. Two patients with K19-positive HCC were lost to follow-up. Among the remaining 7 patients with K19-positive HCC and complete follow-up data, 6 had tumour recurrence and 6 (85.7 %) died. Patients with K19-positive HCC had significantly decreased mean recurrence-free (RFS) ($p<0.001$) and overall survival (OS) ($p=0.002$) (Figure 4) compared to those with K19-negative HCC. EpCAM or CD117 positivity did not show any significant correlation with neither RFS nor OS. When K19 and EpCAM co-expression was examined, patients with K19-positive/EpCAM-positive HCC were characterized by significantly decreased RFS ($p<0.001$) and OS ($p=0.001$) compared to those with K19-negative/EpCAM-negative HCC. Although numbers are very small to draw robust conclusions, it is of interest that patients with HCC showing K19 and EpCAM co-expression ($n=3$)

had worse median RFS and median OS time compared to patients with HCC exhibiting K19-positivity only (n=5) (Log rank test, $p=0.006$ and $p=0.001$, respectively).

a. Univariate analysis

In univariate analysis, microvascular invasion ($p=0.004$) (Figure 5) and advanced TNM stage \geq III ($p=0.047$) significantly correlated with decreased RFS. There was no relationship between tumour size $>5\text{cm}$ ($p=1.00$), histological grade ≥ 3 ($p=1.00$), previous treatment (TACE or RFA) ($p=0.133$) and RFS. The presence of microvascular invasion was significantly associated with OS ($p=0.029$) (Figure 5). Tumour size $>5\text{cm}$ ($p=0.56$), advanced histological grade ($p=0.95$), TNM stage \geq III ($p=0.089$), and previous treatment (TACE or RFA) ($p=0.635$) did not correlate significantly with OS.

b. Multivariate analysis

All variables that showed significant correlation with OS and RFS were included in the multivariate model building strategy. Cox-regression analysis revealed that K19- positivity was independently associated with RFS [Odds ratio (OR)=7.84, 95% confidence interval (CI)=2.658-22.912; $p<0.001$], whereas the presence of microvascular invasion and TNM stage \geq III did not show independent association with RFS (Table 2). Similarly, K19-positivity was the only independent prognostic factor of OS (OR=3.845, 95% CI=1.401-10.549; $p=0.009$, Table 3).

Discussion

It is a well-established theory that human HCCs usually emerge during the process of multistep carcinogenesis [34]. Mature hepatocytes may transform creating pre-malignant lesions, namely dysplastic foci and dysplastic nodules, that may give rise to well differentiated early stage HCCs (early HCCs) [35]. Moreover, there is growing evidence that some HCCs derive from HPCs [34]. Primary liver carcinomas of combined type (combined HCC and cholangiocarcinoma) and HCCs that express progenitor cell markers are now recognised as examples of such alternative carcinogenic pathways [5, 36].

Two different theories have so far been expressed with regards to the presence of progenitor cell features in a tumour. Either, the cell of origin is a progenitor cell (maturation arrest theory) or alternatively, tumours de-differentiate and acquire progenitor cell features during carcinogenesis (dedifferentiation theory) [7, 37-39]. The presence of progenitor cells in early premalignant lesions, such as dysplastic foci, is a strong argument in favour of a progenitor cell origin as opposed to dedifferentiation at a later stage [38].

The adverse prognosis of HCCs expressing HPC/stem cell markers may partly be attributed to the relationship between this subset of HCCs with invasion and metastasis-related gene expression. Up-regulation of invasion and metastasis-related genes, such as epithelial-mesenchymal transition (EMT) genes [7, 9], *VIL2* (encoding ezrin) [7, 40], *PLAUR* (uPAR: urokinase plasminogen activator receptor) [7], and *CD44* [7] has been demonstrated in HCCs with HPC features. Moreover, an association between high expression

levels of HPC markers in HCCs and tumour angiogenesis has recently been reported [4]. HCCs expressing "stemness"-related proteins are also characterized by increased telomere length, increased expression of hTERT and shelterin complex proteins, and increased chromosomal instability compared to conventional HCCs as shown in a recent study [11].

There are strong indications that K19 may be used as an HPC marker of prognostic significance [10, 17, 18]. Recently, a progenitor cell-derived HCC model was established in rats, which was characterized by a K19-positive gene signature that accurately predicted tumour recurrence and patient survival in human HCC [3]. Along these lines, an extensive gene-expression profiling study demonstrated the presence of a "hepatoblast-subtype" of human HCCs characterised by K19 expression [4]. There is growing evidence that K19-positivity is associated with increased expression of invasion-related proteins, both at protein and mRNA level [9, 19]. It still remains uncertain whether K19 expression implies that K19-positive HCCs actually carry stemness functions, as shown for the K19-positive ductular reactions of the regenerating liver, or that this expression is merely an epiphenomenon of poor differentiation [39].

Several studies have reported K19 protein expression in HCCs varying from 4.1% to 22.1% [5, 9, 12-15, 17, 20, 22, 36, 39, 41-46]. K19 expression has been correlated with poor histological differentiation [14, 15, 20], macro- and microvascular invasion [5, 9], advanced tumour stage and lymph node metastasis [13]. Recurrence-free survival and overall survival of patients with

K19-positive HCC has been shown to be reduced following surgical resection [4, 21], radio-frequency ablation [43] or liver-transplantation [22]. In addition, K19 immunopositivity in needle biopsies of HCCs has been recently identified as an independent predictor of overall patient survival [46]. There is limited evidence indicating increased resistance to chemotherapy in HCCs that express HPC markers, including K19 [45].

In our study, K19, EpCAM and CD117 expression were examined in a series of HCCs with high incidence of HBV infection, which remains a major aetiological factor of chronic liver disease in Greek population [47]. The expression of K19 in our study group was 10.11% and showed no correlation with baseline characteristics such as tumour size, background chronic liver disease aetiology and histological grade. Among the baseline tumour characteristics, microvascular invasion was the only parameter that tended to correlate with K19 tissue expression, a finding that is concordant with previous evidence on the positive correlation of K19 expression with tumour angiogenesis and invasion [5, 9, 19]. The correlation of K19 with microvascular invasion did not reach statistical significance ($p=0.054$) most likely due to type II statistical error; examining a larger number of HCC could have shown a significant association. In univariate analysis, advanced TNM stage was correlated with RFS, while microvascular invasion with both RFS and OS. However, multivariate analysis indicated that K19-positivity was the only independent prognostic factor for both OS and RFS. Our findings highlight the prognostic importance of K19-positivity in a Caucasian patient cohort, as most relevant data to date come from Asian studies [5, 9, 12-14,

17, 20, 36, 39, 42-45], and are in keeping with those of a recent prospective study from Belgium on K19 immunohistochemical expression in 242 HCC from Caucasian patients [19] .

Similarly, EpCAM has previously been identified as a marker of oval cells in rodent liver and stem/progenitor cells of adult human liver [25, 33, 48-53]. Recent studies, have shown that isolation of EpCAM(+) tumour cells from peripheral blood strongly correlates with prognosis in patients with HCC [54, 55]. In a study by Yamashita et al [33], EpCAM and alpha-fetoprotein (AFP) expression was used to classify HCCs in four different subtypes with different prognosis and specific activated pathways depending on tumour cell origin. The categories proposed were EpCAM(+)/AFP(+) HCC as hepatic stem cell-like HCC (HpSC-HCC), EpCAM(+)/AFP(-) HCC as bile duct epithelium-like HCC (BDE-HCC), EpCAM(-)/AFP(+) HCC as hepatocyte progenitor-like HCC (HP-HCC), and EpCAM(-)/AFP(-) HCC as mature hepatocyte-like HCC (MH-HCC). The same study showed that other HPC markers such as K19 and CD117 were more abundantly expressed in HpSC-HCC. EpCAM(+)/AFP(+) HCC (HpSC-HCC) were characterized by poor prognosis whereas EpCAM(+)/AFP(-) HCC (MH-HCC) exhibited more favourable outcomes. In our study, isolated EpCAM expression did not significantly correlate with patient RFS or OS. This finding corroborates previous data showing that EpCAM expression is present in both hepatic stem-like and mature hepatocyte-like HCCs that are characterized by opposing clinical outcomes [33]. In our series K19/EpCAM co-expression in HCC was associated with worse RFS and OS compared to that of patients with HCC expressing K19

alone. However, this finding needs to be further assessed in a larger number of HCC as in our study group K19/EpCAM co-expression was only seen in 3/78 HCC. CD117 was only expressed in a small percentage of tumour cells in only 3.7% of HCC and showed no significant association with prognostic features.

In conclusion, our study showed that K19 immunopositivity is an independent predictor of RFS and OS in a cohort of Greek patients with HCC and could be used to stratify HCC according to tumour aggressiveness. Our study supports the prognostic role of K19 independently of other clinico-pathological parameters. Inclusion of K19 expression, as a marker of tumour aggressiveness in HCC, independently of tumour grade and stage, may increase the predictive value of the currently used scoring systems. The recent association between K19 expression and the epidermal growth factor receptor (EGFR) pathway in HCC [44] suggests a possible role for therapeutic use of EGFR inhibitors and may open the way for a more personalised treatment approach in HCC patients.

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Legends to figures

Figure 1. HCC grade 2: Few K19-positive tumour cells (arrows). K19-positive ductules at tumour periphery (black arrowheads) and intratumoural ductular cells (white arrowheads) are noted (x200)

Figure 2. Serial sections of HCC positive for K19 (A) and EpCAM (B). Some positive tumour cells show morphological features of stem cells with smaller, dark nuclei and scant cytoplasm (arrow); C & D: Serial sections of HCC positive for K19 (C) but negative for EpCAM (D), chromagen DAB (x200)

Figure 3. Serial sections of well differentiated HCC (grade 2), trabecular pattern: A. H-E (x200), B. Keratin 19 immunoreactivity in >90% of tumour cells (x200), C: Moderately differentiated HCC (grade 3), solid pattern with EpCAM-positivity in >90% of tumour cells (x200), D. A tumour cell (arrow) and mast cells (arrowhead) positive for CD117, chromagen DAB (x400). CD117-positive HCC showed isolated immunopositivity in 5% of tumour cells.

Figure 4. A: K19 expression in relation to overall survival of HCC patients (Kaplan-Meier curves, log-rank test $p=0.002$); **B:** K19 expression in relation to recurrence-free survival of HCC patients (Kaplan-Meier curves, log-rank test $p<0.001$)

Figure 5. A: Microvascular invasion in relation to overall survival of HCC patients (Kaplan-Meier curves, log-rank test, $p=0.029$); **B.** Microvascular

invasion in relation to recurrence-free survival of HCC patients (Kaplan-Meier curves, log-rank test, $p=0.004$)

Table 1. HCC patients' characteristics and clinico-pathological parameters in correlation with K19 protein, EpCAM and CD117 expression.

FEATURE	K19+ n/N	K19- n/N	p	EpCAM+ n/N	EpCAM- n/N	p	CD117+ n/N	CD117- n/N
Age (years, mean±SD)	67±12	67±11	0.961	70.4±8.2	65±11	0.298	70.5	67±1
Male gender	5/9	62/80	0.385	6/12	53/66	0.035	3/3	59/78
Cirrhosis	5/7	41/66	1.000	4/9	39/55	0.059	1/2	40/65
HBV	2/7	28/65	0.429	6/8	24/55	0.118	1/3	29/63
HCV	2/7	19/65	1.000	1/8	20/55	0.155	0/3	21/63
Alcohol	0/7	4/65	0.985	0/12	4/45	0.257	0/1	4/64
≥3 nodules	3/7	17/68	0.331	3/11	15/57	0.638	1/3	17/67
Tumour size > 5cm	5/7	32/65	1.000	7/12	25/54	0.294	1/3	35/65
Tumour grade ≥ 3	4/9	38/80	0.862	6/12	33/66	1.000	1/3	38/78
Microvascular invasion	7/8	32/62	0.054	6/11	30/53	0.901	2/3	35/62
Portal vein thrombosis	3/5	15/42	0.357	2/6	15/36	0.082	2/2	16/43
Capsular invasion	2/4	25/50	0.695	3/8	21/40	0.439	0/2	27/48
Lymph node metastasis	1/7	2/59	0.290	0/12	3/51	0.464	0/3	2/57
Distant metastasis	0/3	6/33	1.000	0/4	5/28	0.358	0/3	6/31
TNM stage ≥ III	5/8	33/69	0.481	3/11	32/58	0.091	2/3	35/69

n: number of cases with the feature, N: total number of cases with available information on the feature, SD: standard deviation, K19: keratin 19, EpCAM: Epithelial cell adhesion molecule, +: positive, -: negative immunohistochemical expression (p Values in CD117 were not included due to small number of positive cases)

Table 2. Cox-regression analysis for recurrence-free survival of HCC patients

	Sig.	OR	95.0% CI for OR	
			Lower	Upper
Keratin 19 protein expression	0.000	7.804	2.658	22.912
Microvascular invasion	0.099	2.194	0.863	5.576
TNM Stage \geq III	0.390	1.461	0.615	3.468

Sig.: significance, OR: odds ratio, CI: confidence interval

Table 3. Cox-regression analysis for overall survival of HCC patients

	Sig.	OR	95.0% CI for OR	
			Lower	Upper
Microvascular invasion	0.208	1.832	0.715	4.696
Keratin 19 protein expression	0.009	3.845	1.401	10.549

Sig.: significance, OR: odds ratio, CI: confidence interval